

Research Paper

Partial Müllerian Duct Retention in *Smad4* Conditional Mutant Male Mice

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Abstract

Müllerian duct regression is a complex process which involves the AMH signalling pathway. We have previously demonstrated that besides AMH and its specific type II receptor (AMHRII), BMPR-IA and *Smad5* are two essential factors implicated in this mechanism.

Mothers against decapentaplegic homolog 4 (*Smad4*) is a transcription factor and the common *Smad* (co-*Smad*) involved in transforming growth factor beta (TGF- β) signalling pathway superfamily. Since *Smad4* null mutants die early during gastrulation, we have inactivated *Smad4* in the Müllerian duct mesenchyme. Specific inactivation of *Smad4* in the urogenital ridge leads to the partial persistence of the Müllerian duct in adult male mice. Careful examination of the urogenital tract reveals that the Müllerian duct retention is randomly distributed either on one side or both sides. Histological analysis shows a uterus-like structure, which is confirmed by the expression of estrogen receptor α . As previously described in a β -catenin conditional mutant mouse model, β -catenin contributes to Müllerian duct regression. In our mutant male embryos, it appears that β -catenin expression is locally reduced along the urogenital ridge as compared to control mice. Moreover, the expression pattern is similar to those observed in control female mice. This study shows that reduced *Smad4* expression disrupts the Wnt/ β -catenin signalling leading to the partial persistence of Müllerian duct.

Key words: *Smad4*, Müllerian duct, conditional knockout, β -catenin.

Introduction

At the indifferent stage, the foetus possesses a bipotential gonad and two pairs of ducts: the Müllerian duct and the Wolffian duct. During development, the Wolffian duct forms first and elongates in an anteroposterior direction. After its formation, the Müllerian duct grows from rostral to caudal and joins the urogenital sinus. In the female, the Müllerian duct gives rise to the oviduct, uterus and upper part of the vagina. Under the influence of testosterone the Wolffian duct differentiates into the vas deferens, epididymis and seminal vesicle in males [1].

Müllerian duct regression is an active developmental process involving anti-Müllerian

hormone (AMH) and its specific receptor AMHR-II [2, 3]. AMH is a glycoprotein that belongs to the transforming growth factor beta (TGF- β) family [4]. It is secreted by foetal Sertoli cells during male sexual differentiation. Like the other members of the TGF- β family, AMH transduces its effects through two transmembrane serine/threonine kinase receptors type I and II, and two types of cytoplasmic effectors, specific *Smads* (R-*Smads*), and a *Smad* protein common to all ligands, *Smad4* [5]. The signalling pathway downstream of AMH in the Müllerian duct is now well documented: it involves mainly the bone morphogenetic protein receptor type IA (BMPR-IA, also named ALK3) and *Smad1/Smad5* proteins [6, 7].

However, although Smad4 is described as the common Smad, several studies suggest that BMP and TGF- β signalling can be independent of Smad4 [8, 9].

Smad4 is involved in numerous developmental processes including early development, cell growth, differentiation and pathologies like cancer. Because of its major role during gastrulation [10, 11], *Smad4* deficient embryos cannot form preventing post-natal analysis of Smad4 functions. In addition, haploid loss of *Smad4* in mice triggers gastric cancer [12]. To circumvent this, mice bearing a *Smad4* floxed allele were produced to allow the generation of different conditional mutant mice [13-15].

Among the numerous important functions exerted by BMP family members during development, Müllerian duct regression is a very specific process, which occurs exclusively in males between E13.5 and E15.5 in mice [2]. The aim of this study is to determine the potential involvement of Smad4 during this process. We have designed a specific deletion of *Smad4* in the Müllerian duct using the Cre-lox system by crossing the *Amhr2-Cre* [*Amhr2^{tm3(cre)Bhr}*] mouse line with mice bearing the *Smad4* floxed allele [*Smad4^{tm2.1Cxd}*] [6, 15]. We then analysed the phenotype of the conditional mutant males both macroscopically and at the cellular level. We show that males display a consistent phenotype indicating that Smad4 might play a role in the completion of Müllerian duct regression.

Materials and Methods

Mice

Amhr2-Cre (*Amhr2^{tm3(cre)Bhr}*) and *Smad4* floxed mice (*Smad4^{tm2.1Cxd}*) were maintained on a C57BL/6J; 129/SvEv mixed genetic background as previously described [6, 15]. *Amhr2^{+cre}* mice were crossed with *Smad4^{f/f}* to generate *Amhr2^{+cre}; Smad4^{f/f}* mice. These mice were then mated with *Smad4^{f/f}* female mice to generate the *Amhr2^{+cre}; Smad4^{f/f}* conditional knockout mice (*Smad4* cKO, noted *Smad4^{Δ/Δ}*). The *Smad4^{f/f}* littermates were used as control mice. Genotyping was performed on DNA from tail biopsies or amniotic sac using standard PCR protocols. Mice used for the experiments were genotyped twice, at weaning and at sacrifice respectively, in order to confirm the genotype.

RNA isolation and PCR amplification

For regular PCR, total RNAs from E14.5 mesonephri were isolated using the RNeasy Plus Mini kit (Qiagen) according to the manufacturer's instructions. Reverse transcription was performed in a total of 20 μ l with the High capacity RNA-to-cDNA™ Kit for RT-PCR (Life Technologies) using 125 ng total

RNA. The samples were incubated 1 h at 37°C and inactivation was performed for 5 min at 95°C. PCR was performed using HotStarTaq Plus DNA Polymerase (Qiagen) according to the manufacturer's instructions. The amplification conditions were 95°C for 5 min, followed by 35 cycles at 94°C for 45 s, 58°C for 45 s, and 72°C for 45 s, with a final extension at 72°C for 10 min. The amplified PCR fragments were analysed on 2% agarose gels and visualized with ImageQuant 350 (GE Healthcare).

Primer sequences. *Amhr2-Cre*: PTMWbpA-s: CGC ATT GTC TGA GTA GGT GT. mAmhr2 E5-a: GAA ACG CAG CTC GGC CAG C. *Smad4*: Q-m-Smad4-1368-s: CAG-CCT-CCC-ATT-TCC-AAT-C. Q-m-Smad4-1694-a: GCC-AGC-TTC-TCT-GTC-CAG-GT.

For real time PCR, 200 ng of total RNAs from E14.5 mesonephri were isolated using Arcturus PicoPure RNA isolation kit (Thermo Fisher Scientific) and reverse transcription was performed using iScript cDNA synthesis kit (Bio-Rad) following the manufacturer's instructions. The synthesis was achieved at 42°C for 30 min and heat inactivated at 85°C for 5 min. Real time PCR was set up with iTaq Universal SYBR Green supermix (Biorad) and run in a Bio-Rad CFX384 apparatus. Samples were analyzed in duplicates with one-tenth dilution of the cDNAs. The PCR program was 95°C for 3 min for initial denaturation, then 40 cycles as follows 95°C for 10 sec and 60°C for 30 sec. The melting curve was generated by heating from 65°C to 95°C. Standard curves were generated with increasing dilutions of a control cDNA template. Relative gene expression was normalized to an endogenous control gene (*Rpl13a*).

Primer sequences. *Smad4*: Q-m-Smad4-1482-s: ACT-GTG-GAT-GGC-TAT-GTG-GAT. Q-m-Smad4-1594-a: CCT-TTG-CCT-ATG-TGC-AAC-CT. *Rpl13a*: Q-m-Rpl13a-458-s: CAT-GAG-GTC-GGG-TGG-AAG-TA. Q-m-Rpl13a-573-a: GCC-TGT-TTC-CGT-AAC-CTC-AA.

Histology, Immunohistochemistry (IHC) and Immunofluorescence (IF)

Reproductive tracts were collected from male mice at 3 weeks, 3 months and 12 months in PBS and visualized under a Zeiss stereomicroscope Stereo Discovery V8 connected to an Axiocam ICc3 camera. Uteri from mature female mice in oestrus were collected to serve as controls. Embryos at E14.5 were dissected out in PBS and the amniotic sac was used for genotyping. All samples were fixed overnight at 4°C in 4% paraformaldehyde before the procedure of dehydration and embedding in paraffin. 5 μ m sections were rehydrated and then the antigen was unmasked (Antigen Unmasking Solution, Vector

Laboratories).

For IHC, the endogenous peroxidase activity was quenched and a blocking solution (1.5% goat serum or MOM Mouse Ig Blocking reagent (Vector Laboratories), 1x PBS and Avidin D, Vector Laboratories) was applied for 60 min. Sections were incubated overnight at 4°C with the primary antibody diluted into a solution containing 1.5% goat serum in PBS or a MOM protein concentrate (Vector Laboratories) and Biotin (Vector Laboratories). After several washes with PBS, the secondary goat anti-rabbit or a MOM biotinylated anti-mouse antibody (Vector Laboratories) was added onto the sections for 60 min. The signal was amplified using the ABC kit (Vector Laboratories), the DAB substrate (Sigma) was applied and the sections were counterstained with Harris hematoxylin (Leica Microsystems).

For IF, specimen were blocked in blocking buffer (1x PBS, 2% BSA, 0.3% Triton™ X-100) for 60 min. A primary antibody diluted into the antibody dilution buffer (1x PBS, 1% BSA, 0.3% Triton™ X-100) was applied overnight at 4°C. After 3 washes in PBS, sections are incubated with the secondary antibody (Life Technologies, AlexaFluor 594, 1:500) in the antibody dilution buffer. After 3 washes in PBS, VectaShield Hard Set Antifade Mounting Medium with DAPI (Vector Laboratories) is added before coverslipping.

The primary antibody used were as follow: a rabbit monoclonal anti-Smad4 C-terminal antibody (Abcam, ab40759, 1:100), a rabbit polyclonal anti-estrogen receptor α antibody (Santa Cruz, sc7207, 1:200), a rabbit monoclonal anti E-cadherin antibody (Cell Signaling, 3195, 1:400), a rabbit monoclonal anti-LEF1 antibody (Cell Signaling, 2230, 1:400), a rabbit monoclonal anti β -catenin antibody (Abcam, ab32572, 1:800) and a mouse monoclonal anti α -SMA antibody (Sigma, T7451, 1:800).

Ethical Statement

Housing and care, method of euthanasia and experimental protocols were conducted in accordance with the recommendations of the French Accreditation of Laboratory Animal Care and in compliance with the NIH Guide for Care and Use of Laboratory Animals. The animal facility is licensed by the French Ministry of Agriculture (agreement C35-238-19). All animal experiments were supervised by Dr. Soazik Jamin and Dr. Fabrice Petit (agreement delivered by the French Ministry of Agriculture for animal experiment # 92-299 and # A92-313, respectively). Animals were sacrificed with CO₂. All efforts were made to minimize animal suffering.

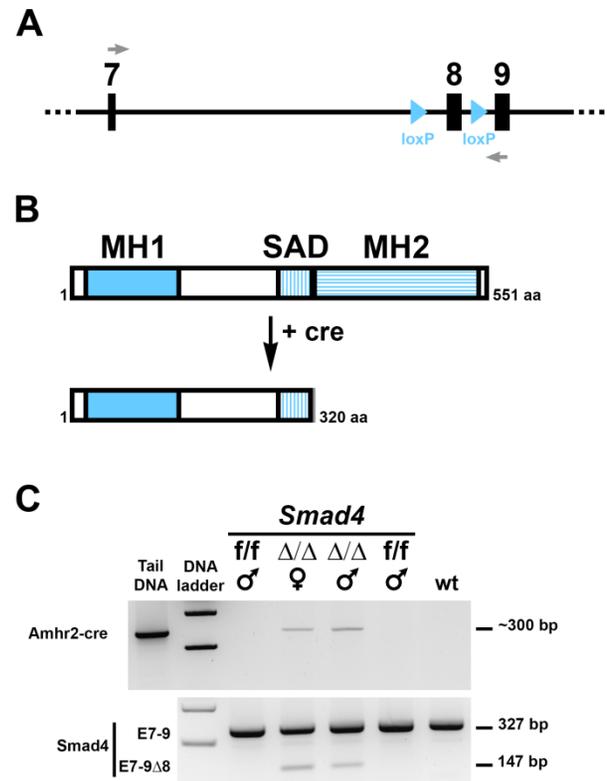


Figure 1. Conditional deletion of exon 8 of the *Smad4* gene in the gonadal ridges of 14.5 dpc *Smad4* cKO embryos. (A) schematic diagram of the *Smad4* conditional allele (exon 7 to 9) showing lox P sites flanking exon 8. Grey arrows represent the primers used to distinguish the full length *Smad4* mRNA from the $\Delta 8$ *Smad4* mRNA. (B) Like other Smad proteins, Smad4 possesses two conserved regions, MH1 and MH2 (MAD homology 1 and 2) separated by a linker region SAD (Smad4 Activation Domain). MH1 domain exhibits sequence-specific DNA-binding, while MH2 is responsible for heteromerization and transactivation functions. Deletion of *Smad4* exon 8 using a Cre-expressing mouse leads to the production of a truncated Smad4 protein missing the MH2 domain. (C) RT-PCR on Cre (top panel) and *Smad4* (bottom panel) products shows that Cre-expressing mice (*Smad4^{Δ/Δ}*, noted Δ/Δ) possess the full-length (327 bp) and the truncated (147 bp) RNA. *Smad4^{f/f}* (*f/f*) and wt mice were used as controls. Tail DNA from a Cre-expressing mouse was used as a positive control for the Cre.

Results

To address the role of Smad4 during Müllerian duct regression, we have generated conditional mutant male mice lacking Smad4 in the mesenchyme surrounding the epithelium of the duct. The *Smad4* conditional allele was generated by flanking exon 8 with 2 loxP sites (Fig. 1A) [15]. Specific deletion of floxed *Smad4* alleles in the mesenchyme of the urogenital ridge is accomplished by using a Cre line expressing the recombinase under the control of *Amhr2* (*Amhr2-Cre*) [6]. We crossed *Amhr2^{+/-cre}*; *Smad4^{+/-f}* males with *Smad4^{f/f}* females to generate *Amhr2^{+/-cre}*; *Smad4^{f/f}* male mice (*Smad4^{Δ/Δ}*). Deletion of exon 8 leads to the expression of a truncated Smad4 protein, which lacks the MH2 domain (Fig. 1B) and therefore cannot bind other Smad proteins, transcription factors, coactivators and corepressors [16]. We first checked *Smad4* deletion in the Müllerian duct using RT-PCR on mesonephri. We found that in

addition to the wild-type allele (327 bp) a mutated allele (147 bp) was generated (Fig. 1C, bottom panel). This short PCR product indicates that the recombination occurred in the desired tissue. As a control, we show that the corresponding individuals express Cre recombinase driven by the *Amhr2* promoter (Fig. 1C, top panel).

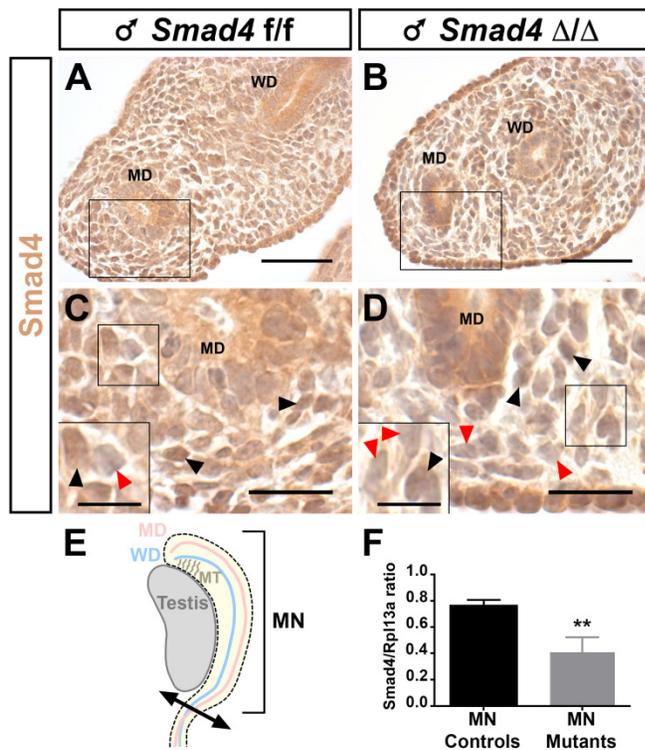


Figure 2. *Smad4* expression in the E14.5 male mouse urogenital ridge. Paraffin-embedded sections are incubated in presence of anti-*Smad4* Ct antibody. (A, C) Nuclear *Smad4* expression is detected in the mesenchymal compartment surrounding the Müllerian duct and Wolffian duct of control *Smad4^{f/f}* embryos. (B, D) Partial *Smad4* expression is observed in the mesenchymal cells of the urogenital ridge of mutant *Smad4^{Δ/Δ}* embryos. (C, D) High magnifications of the respective marked areas in A and B. Inserts in the bottom left corner are higher magnifications of the marked area. Black arrowheads point to *Smad4* expressing cells and red arrowheads point to non-expressing cells. (E) Schematic representation of the embryonic testis and adjacent mesonephros (MN), which was used for RNA extraction and subsequent real-time PCR. (F) The level of *Smad4* expression was measured using real-time PCR. *Rpl13a* was used to normalize the results. Data were analyzed using paired t-test followed by Mann-Whitney test. ** $p < 0.01$. *Smad4* expression is significantly decreased in conditional mutant mesonephri compared to controls. Scale bars, 20 μm (A- D) and 10 μm (inserts in C and D). MD, Müllerian duct; MN, mesonephros; MT, mesonephric tubules; WVD, Wolffian duct.

Since the *Smad4^{Δ/Δ}* mice express a C-terminally truncated *Smad4* protein, we used a *Smad4* antibody directed against the C terminal part of *Smad4* to discriminate the cells expressing full-length *Smad4* from those that express the truncated protein by immunohistochemistry. As expected, at embryonic day 14.5 (E14.5) *Smad4* is widely expressed in the urogenital ridge including the Wolffian duct and Müllerian duct (Fig. 2A and C). In *Smad4^{Δ/Δ}* embryos, the full-length *Smad4* protein level is only partially reduced since some mesenchymal cells still express *Smad4* (Fig. 2B and D). The quantification of *Smad4*

expression was achieved on embryonic mesonephri isolated from three individuals (Fig. 2E). After RNA extraction and reverse transcription, real-time PCR was performed on control and mutant mesonephri. *Rpl13a* expression was used for the internal control. As shown in Fig. 2F, *Smad4* expression was significantly decreased by two fold in mutant mesonephri indicating that the recombination occurred in this organ.

As expected, the generation of *Smad4^{Δ/Δ}* mice is not perturbed by the specific inactivation of *Smad4* in the urogenital ridge and the gonads. *Smad4^{Δ/Δ}* mice are viable but the reproductive functions differ in females and males. Mutant females are infertile (Petit *et al.*, in preparation) and mutant males are fertile. Indeed, the overall reproductive performance of the mutant males was not affected. They regularly have pups and the litter size did not show any significant differences between control (7.2 ± 1.8 , $n=10$) and mutant (7.3 ± 1.9 , $n=24$). The macroscopic and detailed observation of immature (3-week-old) or mature (3- and 12-month-old) *Smad4^{Δ/Δ}* male mice shows that they retain a partial Müllerian duct as compared to control *Smad4^{f/f}* (Fig. 3). The presence of these Müllerian duct remnants is observed in all individuals regardless of age but their number and location may vary from one to another. Indeed, we observed one Müllerian duct remnant on one side (Fig. 3E, F, O, P, Q and R) or two sides (Fig. 3I, J, J', K and L), and two on one side (Fig. 3C, D). They are located anywhere along the vas deferens and they persist throughout the mouse life without apparent alteration. Note that control *Smad4^{f/f}* males never exhibit any partial Müllerian duct remnant (Fig. 3A, B, G, H, M and N). Moreover, *Amhr2^{+/cre}; Smad4^{f/f}* males do not present any Müllerian duct retention (data not shown).

To further characterize the identity of the Müllerian duct remnant, we performed a histological analysis by staining the transverse sections with hematoxylin/eosin on mature mice (Fig. 4). As a control, we used a previously described pseudohermaphrodite male [6] (*Amhr2^{cre/cre}*), in which the disruption of AMH signalling pathway leads to the persistence of the whole Müllerian duct alongside the Wolffian duct (Fig. 4A-C). Normal development of either the vas deferens or the uterus was shown using a *Smad4^{f/f}* male (Fig. 4D) and a *Smad4^{+/Δ}* female (Fig. 4E). We find that the vas deferens of mutant males (Fig. 4F) has the same histological features than the vas deferens from pseudohermaphrodite (Fig. 4A) and control (Fig. 4D) male mice. In addition, they all contain spermatozoa indicating that the testis is fully functional. We also analysed the Müllerian duct remnants, which are in close contact with the vas deferens (Fig. 4G).

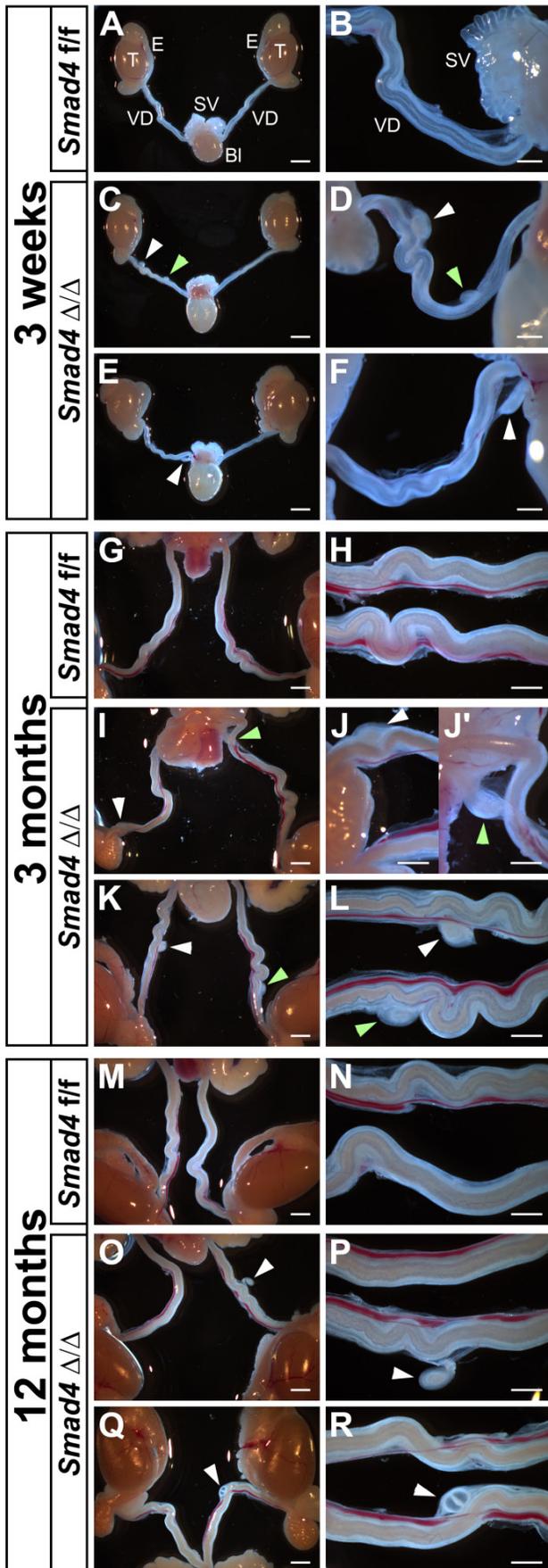


Figure 3. Partial Müllerian duct retention in *Smad4*^{Δ/Δ} mutant mice. The observation of control *Smad4*^{f/f} male mice at different ages shows a normal development of the reproductive tract with a complete regression of Müllerian duct (A, G, M). However, in *Smad4*^{Δ/Δ} mutant reproductive tracts, a persistence of small Müllerian duct parts is observed uni- or bilaterally (C, E, I, K, O, Q, white and green arrowheads). Note that for *Smad4*^{Δ/Δ}, two different mice per age are shown. (B, D, F, H, J, L, N, P, R) Higher magnifications of A, C, E, G, I, K, M, O and Q, respectively. Scale bars, 2 mm (A, C, E, G, I, K, M, O, Q), 1 mm (H, J, J', L, N, P, R) and 500 μm (B, D, F). BI, bladder; E, epididymis; SV, seminal vesicles; T, testis; VD, vas deferens.

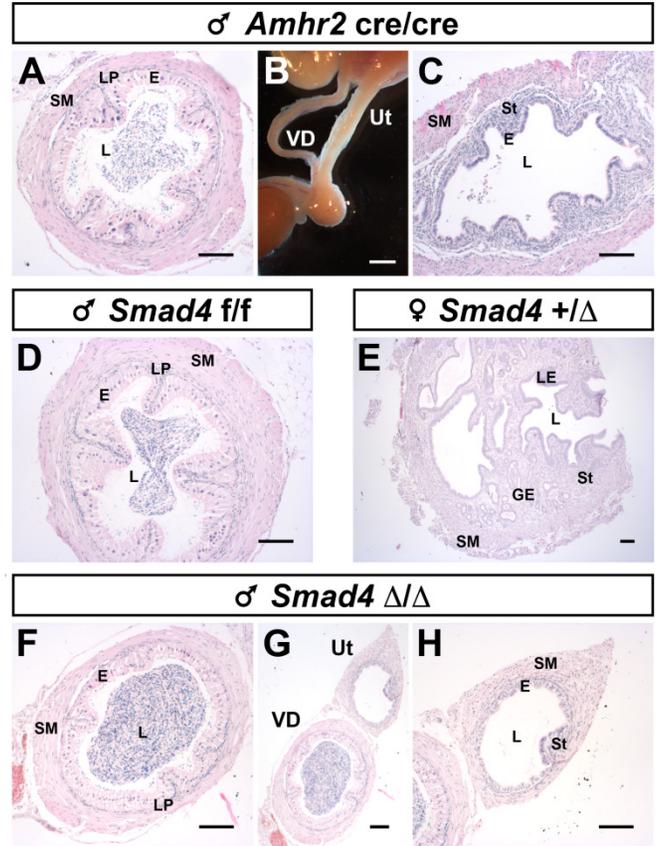


Figure 4. Müllerian duct remnants in *Smad4*^{Δ/Δ} mutant mice develop as uteri. Disruption of the AMH pathway in mature *Amhr2*^{cre/cre} male mice causes retention of the whole Müllerian duct alongside the Wolffian duct. The Müllerian duct and Wolffian duct will develop as uterus and vas deferens, respectively (B). Paraffin embedded sections of vas deferens and uterus stained with hematoxylin/eosin (A, C, D-H). Vas deferens and uterus from *Amhr2*^{cre/cre} male mouse (A, C). Mature *Smad4*^{f/f} male (D) and *Smad4*^{Δ/Δ} female (E) mice present a fully developed vas deferens and uterus, respectively. In mature *Smad4*^{Δ/Δ} mutant mice (F-H), the vas deferens appears normal. Although, the Müllerian duct can develop as a uterus, it is smaller than a normal uterus and often underdeveloped (compare H with E). Scale bars, 2 mm (B) and 100 μm (A, C, D-H). E, epithelium; GE, glandular epithelium; L, lumen; LE, luminal epithelium; LP; lamina propria; SM, smooth muscle; St, stroma; Ut, uterus; VD, vas deferens.

Histologically, all remnants display layers comparable to a normal uterus, including smooth muscle, epithelium and stroma (Fig. 4H versus 4E). Nevertheless, one can notice that the stromal compartment is greatly underdeveloped. We have also checked the identity and responsiveness of the uterus using immunostaining for the estrogen receptor alpha (ERα or NR3A1, Fig. 5B and F). As expected, ERα is highly expressed in the cellular

nucleus of all compartments (epithelium, stroma and myometrium) both in control female and mutant male [17]. On the other hand, the epithelial compartment of the vas deferens does not express ER α (Fig. 5A and E). The different muscle layers both in the vas deferens and in the uterus are marked with α smooth muscle actin antibody (α SMA) (Fig. 5C, D, G and H). This characteristic expression pattern of ER α combined to those of α SMA confirms that the Müllerian duct remnant has a uterus-like structure. As observed in Fig. 4H, 5F and 5H the absence of a glandular epithelium and a developed stromal compartment delineates an immature uterus, which was not exposed to estrogens [18].

As previously shown in a β -catenin mutant mouse model [19], it appears that β -catenin is required for Müllerian duct regression. In addition, during Müllerian duct regression β -catenin and Lef1 accumulates in the nucleus of mesenchymal cells [2]. Therefore, to delineate the possible origin of Müllerian duct remnants in our *Smad4* Δ/Δ mouse model, we performed an immunofluorescence assay on E14.5 urogenital ridge sections to analyse the expression of β -catenin and Lef1 (Fig. 6). To delineate the mesenchyme from the epithelium, we used an antibody directed against E-cadherin. As previously described [20], E-cadherin is uniformly expressed in the coelomic epithelium including the Wolffian duct and the cells bordering the urogenital ridge (Fig. 6C, F, I, L and O). In the urogenital ridge of control female mice, strong membranous expression of β -catenin in the Müllerian duct, Wolffian duct and coelomic epithelium was observed (Fig. 6A). In control male mice, β -catenin protein is not only detected in the epithelium compartment but also in the mesenchyme surrounding the Müllerian duct (Fig. 6D). Since Müllerian duct remnants in mutant male mice can be observed anywhere alongside the Wolffian duct, we have analysed different sections along the urogenital ridge for β -catenin expression (Fig. 6G, J and M). It appears that the majority of sections show a similar expression pattern to the one observed in control male mice (Fig. 6J and M versus 6D). Nevertheless, it is interesting to note that a dramatic reduced expression of β -catenin is observed in some sections (Fig. 6G). Moreover, the expression pattern observed in this section is similar to the one analysed in female (Fig. 6G versus 6A). The expression analysis of the Wnt/ β -catenin target gene Lef1 [21] reveals an absence of immunodetection in sections (Fig. 6B and H), where β -catenin is downregulated in the Müllerian duct mesenchyme (Fig. 6A and G). However, Lef1 is nicely induced in the β -catenin-expressing mesenchyme surrounding the

Müllerian duct (Fig. 6D/E, J/K and M/N). One can note that a reduced expression of β -catenin (Fig. 6J) is correlated with a reduced Lef1 signal (Fig. 6K). This observation suggests that the Wnt/ β -catenin signalling pathway is locally abolished along the Müllerian duct mesenchyme of mutant male mice.

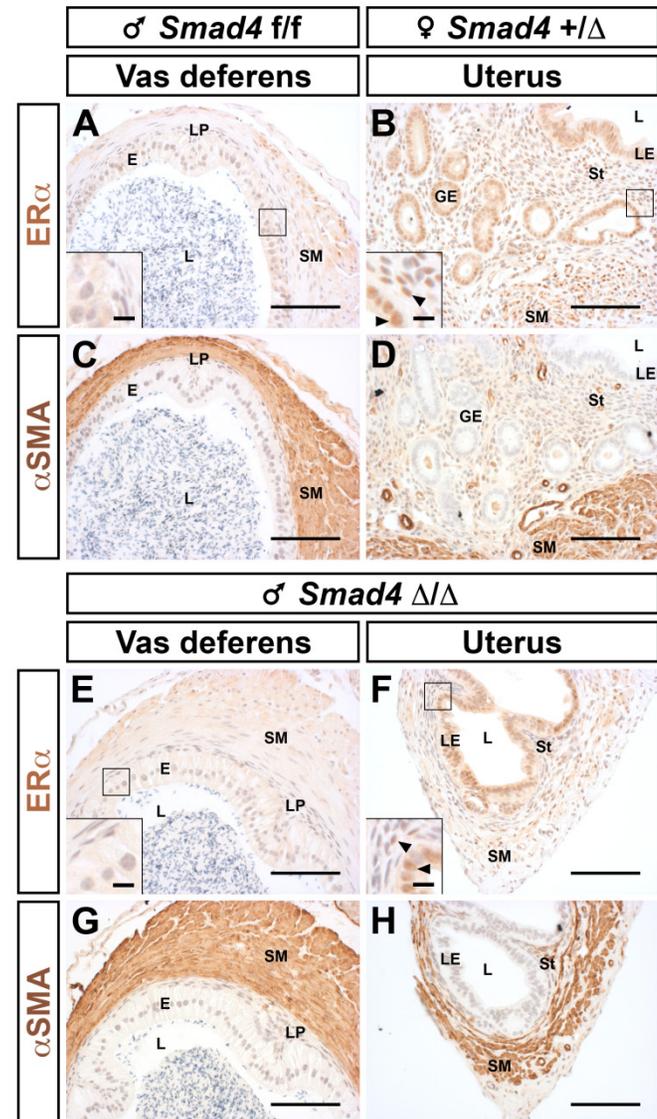


Figure 5. Müllerian duct remnants from *Smad4* conditional mutant male display all histological characteristics of a uterus. Immunostaining for ER α showed that nuclei of the uterine luminal epithelium are marked (B and F) but not nuclei of vas deferens (A and E). The smooth muscle layers are delineated using a α smooth muscle actin (α SMA) antibody (C, D, G and H). Inserts in the bottom left corner of A, B, E and F are higher magnifications of the respective marked area. Arrowheads point to ER α positive cells. Scale bars, 100 μ m (A-H) and 10 μ m (inserts in A, B, E and F). E, epithelium; GE, glandular epithelium; L, lumen; LE, luminal epithelium; LP, lamina propria; SM, smooth muscle; St, stroma.

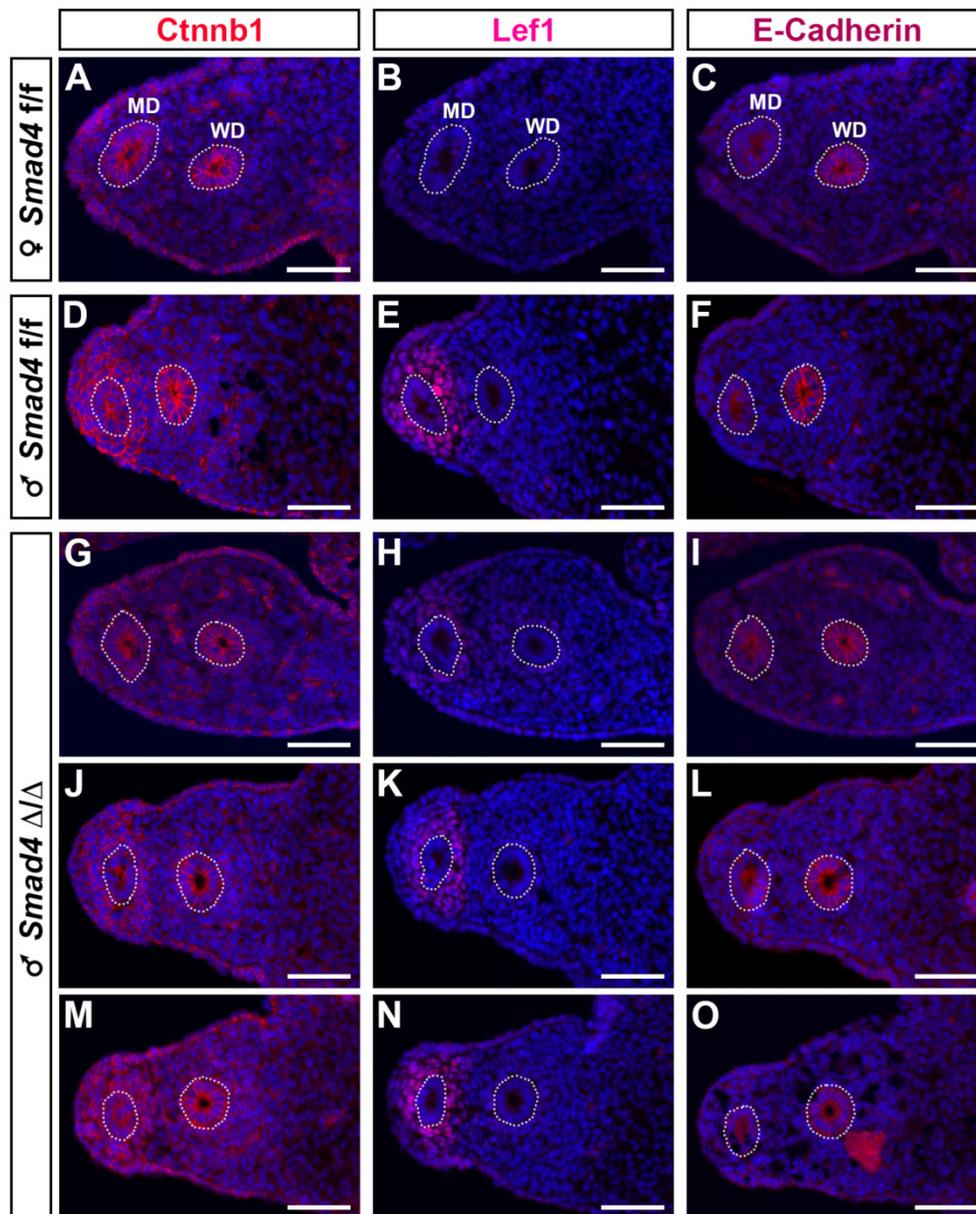


Figure 6. Local reduced expression of β -catenin in the urogenital ridge of the *Smad4 Δ/Δ* mutant male. E14.5 male and female urogenital ridges were analyzed by immunofluorescence (red) for the expression of β -catenin (Ctnnb1, A, D, G, J, M), Lef1, a *Vnt*/ β -catenin target molecule (B, E, H, K, N) and E-cadherin, an epithelium marker (C, F, I, L, O). DAPI staining (blue) was used to delineate the nuclei. In e14.5 *Smad4^{f/f}* female urogenital ridge, β -catenin expression is localized to the cell membrane of the Müllerian duct and Wolffian duct epithelium (A) and Lef1 is not expressed (B). In *Smad4^{f/f}* male, β -catenin is expressed in the Müllerian duct and Wolffian duct epithelium, as well as in the mesenchyme surrounding the Müllerian duct (D), where Lef1 is also strongly expressed. In *Smad4 Δ/Δ* male, different sections along the mesonephros show that β -catenin expression is conserved in the Müllerian duct and Wolffian duct epithelium (G, J, M), but not in the Müllerian duct mesenchymal compartment (G < J < M). In M, the level of expression is similar to the one observed in the control male (D). The expression of β -catenin is correlated with those of Lef1 (H, K, N). Scale bars, 50 μ m. MD, Müllerian duct; WD, Wolffian duct.

Discussion

In this study, we have generated conditional mutant mice lacking *Smad4* in the mesenchyme surrounding the Müllerian duct using a strategy comparable to what we previously performed [6, 7]. Since *Smad4* is the common Smad involved in BMP and activin/TGF- β signalling pathways [5], we hypothesized that it might play a central role in the

pathway downstream of AMH. Therefore, in our *Smad4* conditional knockout mouse model we expected to have a similar phenotype to what was observed in conditional mutants for *Smad1/5* or *Actr1a/Bmpr1a* [6, 7, 22-24]. Conditional double mutants for *Smad1/5* and *Actr1a/Bmpr1a* display a complete Müllerian duct retention leading to the formation of a uterus as it was described for *Amh* and *Amhr2* homozygous mutant male mice [25, 26].

Surprisingly, we observe that *Smad4* deletion in the mesenchyme of the Müllerian duct leads to the partial but consistent retention of Müllerian duct remnants. The mosaic *Smad4* expression could explain the apparent weak involvement of *Smad4* in the AMH pathway and the partial retention phenotype observed. Despite these observations, one can note that the persistence is fully penetrant since it is observed in all animals tested. In the *Amh-Amhr2-Bmpr1a-Smad1/5/8* gene activation cascade, *Smad4* is considered as an important factor helping the phosphorylated *Smads1/5/8* translocate to the nucleus, where the heterodimeric complex *Smad1/5/8-Smad4* transcriptionally activates its target genes [27]. This pathway plays a primordial role for Müllerian duct regression since inactivation of one of these factors is sufficient to maintain the Müllerian duct, which gives rise to a uterus [1]. However, in our study the incomplete *Smad4* gene inactivation shows a moderate effect on Müllerian duct retention. Since *Smad4* deletion is likely mosaic along the Müllerian duct, *Smad4* expression is variable throughout this axis. We hypothesize that, in order to observe a retention, *Smad4* expression must be under a critical threshold, which is reached in one or two regions of the Müllerian duct. A similar observation has been previously reported for *Amh* [28]. These authors show that SF1-binding site mutation in *Amh* promoter leads to a dramatic reduction of *Amh* transcript levels (about 80%). Nevertheless, these homozygous mutant males have no Müllerian duct retention [28].

These observations do not exclude that other factors compensate for the loss of *Smad4* or act in a *Smad4*-independent manner as it is reported for *Trim33* or *DROSHA* [29, 30]. During palate development, TGF- β 3 plays a critical role for epithelial fusion. A recent study has shown that TGF- β 3 signalling involves a *Smad*-dependent pathway both *Smad4* dependent and *Smad4* independent [30]. More specifically, phosphorylated *Smad2* can bind either *Smad4* or *Trim 33*, the two pathways being essential for the correct epithelial fusion during palatogenesis [30]. Another study has shown that miRNA processing is independent of *Smad4* while it depends on the presence of R-*Smads* [29].

As previously shown, the deletion of exon 8 leads to the formation of a truncated *Smad4* protein lacking 198 amino acids in the C-terminal domain, which encompasses the MH2 domain (Fig. 1B) [11, 15]. Several studies have demonstrated that this truncated protein cannot transmit activin/TGF- β or BMP signals showing that this *Smad4* mutation is identical to a null mutation [31-36]. However, since

the truncated *Smad4* protein retains its DNA binding domain and therefore its ability to interact with the *Smad*-binding element [37], one cannot exclude that this mutant protein is biologically active. Indeed, it has been shown that the N-terminal domain is capable of reporter gene transactivation in yeast and mammalian cells [37, 38] and it can interact with CBP/p300 [39]. A dominant negative form is also worth considering since a MH1-containing truncated *Smad4* protein reduces the tumor growth in mice [40]. Moreover, mutations leading to a MH2-truncated *Smad4* have been reported. This protein has been described as a dominant negative form and is found in patients diagnosed with cancer due to an alteration of the TGF- β signalling pathway [41-43]. In the *Smad4* conditional mutant mice mimicking the knockout mice [11, 15], it is possible that the first function of *Smad4* is disrupted in the absence of a fully functional protein and the truncated *Smad4* protein does not interfere with any transcriptional activity. However, in our conditional mouse model, the presence of a truncated protein might be sufficient to perturb the action of other transcription factors. Therefore, diminishing the transcriptional activity of factors involved in the Müllerian duct development probably reduces the effect of *Smad4* gene inactivation in the urogenital ridge.

A similar phenotype of partial Müllerian duct retention was observed in males expressing constitutively active β -catenin (CA β -catenin) [44]. In newborn males expressing and accumulating CA β -catenin in the mesenchyme surrounding the Müllerian duct, focal Müllerian duct remnants were present and persisted through adulthood. This focal Müllerian duct retention was explained by a delayed expression of CA β -catenin due to an *Amhr2-Cre* expression occurring after the Müllerian duct regression has already started [44]. Nevertheless, Behringer's group has shown that the use of *Amhr2-Cre* mice to conditionally inactivate β -catenin in the mesenchyme surrounding the Müllerian duct results in total Müllerian duct retention [19]. Consequently, it appears that β -catenin participates in the process of Müllerian duct regression in male mice. Therefore, it is conceivable that what is thought to be focal Müllerian duct retention in CA β -catenin expressing mice [44] is more likely an incomplete Müllerian duct regression. Like in our study, the location of Müllerian duct remnants varies between individuals. These variations might be due to the mosaic expression of the Cre recombinase driven by *Amhr2* [19]. From these data it seems that *Smad4* can modulate the β -catenin expression in the mesenchyme surrounding the Müllerian duct in male mice and interfere with Müllerian duct regression. Therefore, it

is worth considering that any mutation in *Smad4* leading to an inactive or truncated form may affect the Müllerian duct regression and lead to PMDS. Currently several mutations of *Smad4* resulting in truncated proteins have been shown to affect the functions of other Smad proteins [31, 32, 45].

Abbreviations

AMH, anti-Müllerian hormone; AMHRII, AMH type II receptor; BMP, bone morphogenetic protein; DAB, diaminobenzidine; PBS, Phosphate buffered saline; RT-PCR, reverse transcriptase polymerase chain reaction; Smad, Sma- and Mad-related protein; Wnt, wingless-type MMTV integration site family.

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Competing Interests

The authors have declared that no competing interest exists.

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